

A Novel Assay to Assess the Effectiveness of Antiangiogenic Drugs in Human Breast Cancer

John M. Lyons III, MD,¹ Cathy T. Anthony, PhD,¹ Jessica L. Thomson, PhD,²
and Eugene A. Woltering, MD^{1,3}

¹Department of Surgery, Sections of Surgical Oncology and Surgical Endocrinology, Louisiana State University Health Sciences Center, 200 West Esplanade, Suite 200, Kenner, LA 70065, USA

²USDA ARS Southern Regional Research Center, Baton Rouge, LA, USA

³LSUHSC Stanley S. Scott Cancer Center, New Orleans, LA 70112, USA

Background: Many cytotoxic drugs maintain antiangiogenic properties, but there are no human, tumor-based assays to evaluate their antiangiogenic potential. We used a fibrin–thrombin clot-based angiogenesis model to evaluate the angiogenic response of human breast cancer to various cytotoxic agents commonly used in its treatment.

Methods: Fragments of freshly harvested human breast tumors were embedded in fibrin–thrombin clots and treated with five drugs: adriamycin, taxol, 5-fluorouracil (5-FU), methotrexate, and vincristine. Each treatment group included a mean of 28 fragments (range 16–60). A total of four tumors were tested. Tumor fragments were tested with a single dose of each reagent. Angiogenic initiation, angiogenic growth, and overall angiogenic effect were determined for each treatment group using a previously validated scale.

Results: All four breast cancer specimens tested developed an angiogenic response, sprouting neovessels in vitro in a time-dependent fashion ($r = 0.84$, $P = 0.0007$). Taxol statistically inhibited angiogenesis in all four specimens with decreases in the mean angiogenic initiation, angiogenic growth, and overall effect that were 69%, 81%, and 94% of control values, respectively. Vincristine and 5-FU inhibited the mean overall angiogenic effect by 89% and 82% compared with control, respectively. Adriamycin inhibited overall effect 49%. Methotrexate was less effective.

Conclusion: Freshly harvested breast cancer specimens develop an angiogenic response in a fibrin–thrombin clot-based angiogenesis model and respond to treatment with antineoplastic/antiangiogenic drugs. The antiangiogenic potential of commonly used breast cancer drugs varied among individual tumors. Data obtained from this model is unique and might potentially be used to further enhance the efficacy of cytotoxic regimens and individualize patient therapy.

Current breast cancer treatment regimens are often selected based on the unique characteristics of an individual patient's tumor rather than the characteristics of the entire population of patient tumors.¹ Although cytotoxic drugs remain the cornerstone of adjuvant therapy in breast cancer, assays that help

individualize the selection of chemotherapeutic regimens are relatively limited.

Strategies that predict an individual's response to cytotoxic therapy occasionally include chemoresistance assays and chemosensitivity assays. Such assays test a patient's tumor cells against potential cytotoxic drugs in an in vitro setting. These assays measure either tumor cell proliferation or tumor cell death following exposure to selected chemotherapeutics. The drug concentration and the drug exposure time can be altered in these in vitro assays in an attempt to

Published online September 16, 2008.

Address correspondence and reprint requests to: Eugene A. Woltering, MD; E-mail: ewolte@lsuhsc.edu

Published by Springer Science+Business Media, LLC © 2008 The Society of Surgical Oncology, Inc.

predict a patient's *in vivo* response to the drug. For single agents, the *in vitro* drug sensitivity assays predict clinical response with about 60% accuracy, and *in vitro* resistance assays predict treatment failure with over 90% accuracy.² However, studies that compare the clinical outcomes of assay-selected drug regimens with empirically selected drug regimens have not shown a clear-cut survival benefit.^{3,4}

Many cytotoxic drugs, in addition to directly affecting the tumor cells, possess the ability to inhibit the development of angiogenic blood vessels.⁵ There are very few human-tissue-based assays that can assess the affect of cytotoxic or antiangiogenic agents on the tumor's angiogenic blood vessel development. Woltering et al. described a fibrin–thrombin clot angiogenesis assay which enabled the angiogenic response of freshly harvested human tumor specimens to be evaluated under direct vision.⁶ This assay enables the observer to compare the angiogenic response of control tumor fragments with that of tumor fragments exposed to cytotoxic and other potential antiangiogenic agents. The testing of fresh human tumor specimens against antiangiogenic drugs and cytotoxic agents with antiangiogenic activity may offer another means by which one could identify ineffective drugs and could potentially identify effective antiangiogenic drugs.

We hypothesized that sections of human malignant breast tumors would develop an angiogenic response in a fibrin–thrombin clot angiogenesis assay. We further speculated that treatment of these tumor explants with standard antineoplastic drugs would block the development of neovessels and inhibit the subsequent growth of these neovessels into the fibrin–thrombin clot.

MATERIALS AND METHODS

A total of four breast specimens were obtained from recently removed surgical specimens. These tissues were not needed by the hospital for pathologic analysis and would have otherwise been discarded. Tissues were obtained anonymously with the approval of the Institutional Review Board of Louisiana State University Health Sciences Center (LSUHSC, New Orleans, LA). Due to the anonymity under which these tumors were obtained, researchers were not privy to such pathologic information as the tumor type (other than that this was malignant breast tissue), hormone status, or Her-2/*neu* status. Once obtained, specimens were transported to the lab in Medium 199 (Gibco/Life Technologies, Gaithersburg,

MD). One cubic millimeter fragments of human tumor were prepared using sharp dissection and sterile technique.

Drugs and Drug Treatments

Drugs used in this study included cytotoxic chemotherapeutics which have traditionally been given to patients with breast cancer. A single dose of the following reagents was tested: taxol (8.6 ng/ml), vincristine (1 µg/ml), 5-fluorouracil (22.5 µg/ml), adriamycin (4 µg/ml), and methotrexate (2.8 µg/ml). The concentrations tested in the *in vitro* assay were consistent with clinically achievable drug levels. All drugs, left over from patient treatment, were obtained from an LSUHSC hospital pharmacy. They were stored according to the manufacturers' recommendations.

Fibrin–Thrombin Clot Angiogenesis Assay

Wells in a standard 96-well plate (Corning Inc., Corning, NY) were preloaded with a human thrombin solution (0.8 IU in 2.0 µl/well) (Sigma Chemical Company, St Louis, Missouri). Breast specimens were each sectioned into at least 180 tumor fragments. Fragments were then loaded into the wells randomly allotted to one of six different treatment groups (a control group and one for each of the five drugs). Tumor fragments were randomly placed into the thrombin-loaded wells. This random distribution of tumor fragments was done to ensure that each treatment group had an equal representative of the entire specimen. This was done individually for each of the four breast tumors. Treatment groups contained a mean of 28 fragments (range 16–60). The tissue was then covered with 100 µl clot-forming medium containing fibrinogen (3 mg/ml) and 0.5% Σ-amino caproic acid (Sigma Chemical Company) added to nutrient medium, which consisted of medium-199 and an antibiotic/antimycotic solution (100 U penicillin, 100 U streptomycin sulfate, and 0.25 µg amphotercin/ml; Gibco/Life Technologies, Gaithersburg, MD). This mixture was incubated for approximately 1–2 h in a 6% CO₂, 94% air atmosphere at 37° to allow the fibrin–thrombin clot to form. Once the tissue-containing clot was formed, control wells were treated with 100 µl of a nutrient medium containing 20% fetal bovine serum (GibcoBRL). Test drugs combined with 100 µl medium/fetal bovine serum (FBS) were added to experimental wells at this time. The number of wells in control and experimental treatment groups varied from 16 to –0 with a

mean of 28 wells per treatment group. Total well volume was 200 μ l. The 96-well plates remained in the incubator, and nutrient- or drug-containing medium was changed every seventh day. Individual wells containing tumor fragments were examined under an inverted-phase microscope at 7-day intervals under multiple levels of magnification.

Evaluation of Angiogenesis

Tissue fragments were graded using three parameters: (1) the initiation of angiogenesis (%I), (2) the degree of angiogenic neovessel growth (angiogenic growth), and (3) the overall angiogenic effect. Initiation of an angiogenic response was defined as the development of three or more sprouts around the periphery of the disk, visible at 10 \times magnification. For each treatment group, the percentage initiation was defined as the number of wells exhibiting sprouting/number of wells plated \times 100.

Angiogenic growth and overall angiogenic effect were determined by assessing the angiogenic index (AI) for each well. The AI was defined using a semiquantitative visual rating system devised and validated in our laboratory.^{6,7} Briefly, each tissue fragment was visually rated for the development of vessel formation in all four quadrants. Each tissue quadrant was given a 0–4 rating, depending on the length, density, and percentage of the circumference of sprouts involved in the angiogenic response. Scores of all four quadrants were summed and the summation AI of each well was expressed as a numerical rating from 0 to 16. Angiogenic growth was calculated for each treatment group by taking the mean AI of all wells that initiated an angiogenic response (i.e., only wells with an AI > 0). As this measurement included only wells that initiated an angiogenic response and excluded wells that did not sprout neovessels, this parameter is a quantification of pure angiogenic growth.

The overall angiogenic effect was calculated for each treatment group by taking the mean AI of all wells regardless of whether or not an angiogenic response was initiated. Because this measurement considered all wells including those that did not sprout neovessels (i.e., any wells with an AI \geq 0), this parameter was a measurement that depended on both the degree of new vessel initiation as well as the degree of neovessel growth.

Previous experience using this grading system in human placental vein and human tumor/tissue models showed an excellent correlation between observer scores and more objective ratings such as

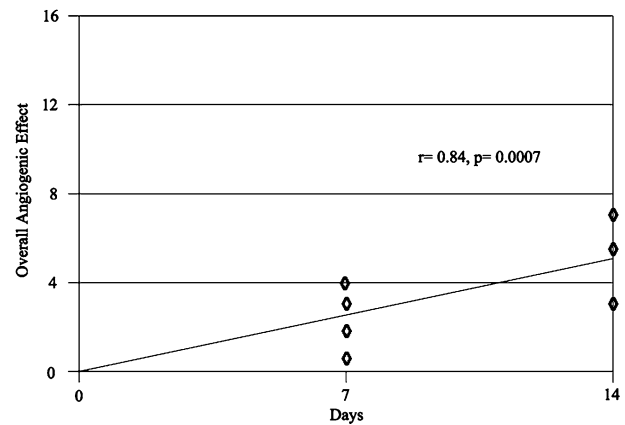


FIG. 1. Breast cancer specimens sprouted neovessels in a time-dependent fashion.

vessel length (mm) or total vessel surface area (mm²) determined by digital image analysis.⁶ Furthermore, AI scores of unbiased observers grading the same wells were statistically similar (Figs. 1–4). The visual rating system is utilized because neovessel growth can be more rapidly evaluated than using more labor-intensive digital image analysis. The neovessel sprouts in these assays have lumens, interconnect, and have all of the attributes of human capillaries. Transmission electron microscopy has been used to confirm that placental vein neovessel sprouts are endothelial in nature. These neovessels exhibit Weibel–Palade bodies and possess tight junctions, detectable on electron microscopy.⁸ The endothelial nature of these sprouts has also been confirmed with immunohistochemical stains for factor VIII.⁶

Statistics

Three different measurements were of interest, each requiring different statistical analysis. Initiation of angiogenesis (%I) was compared between the control group and the treatment groups using a χ^2 test of association. If the overall table value was significant, pairwise comparisons of the treatment groups to the control group were performed using either a χ^2 test of association or Fisher exact test with Bonferroni correction factors applied to the nominal level to maintain an overall type I error rate of 0.05.

Analysis of variance (ANOVA) was used to test for differences among group means for the overall angiogenic effect data. If the *F* test was significant, then Dunnett's test was used to make post hoc pairwise comparisons between the means of the control group and the treatment groups. Due to the

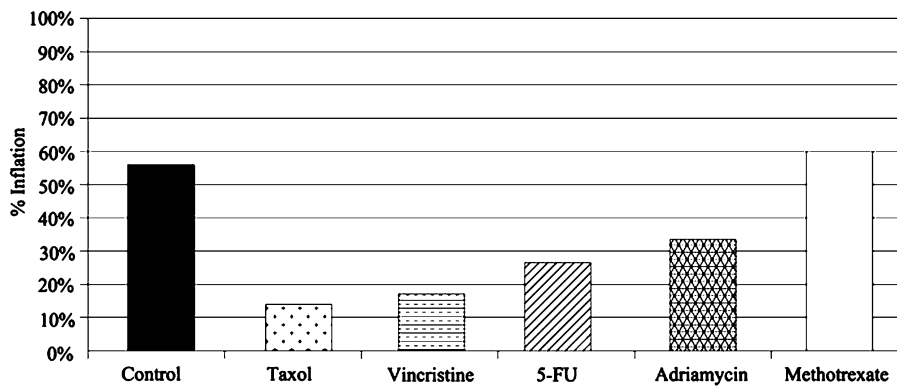


FIG. 2. The effect of antineoplastic drugs on angiogenic initiation in human breast cancer. Results for each treatment group were calculated as: total number of wells that developed angiogenic spouts for all four specimens/total number of wells for all four specimens \times 100.

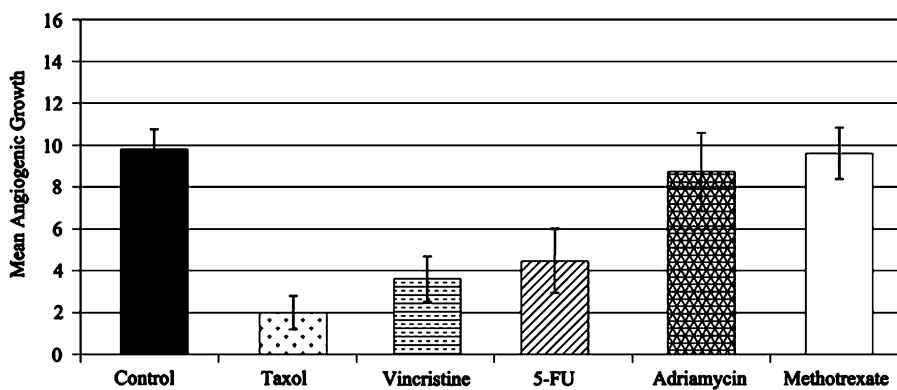


FIG. 3. The effect of antineoplastic drugs on pure angiogenic growth in human breast cancer. Results for each treatment group were determined by calculating the mean angiogenic index (AI) of all wells that developed neovessels (i.e., those with an AI $>$ 0). Y-error bars indicate ± 2 standard error on the mean (SEM).

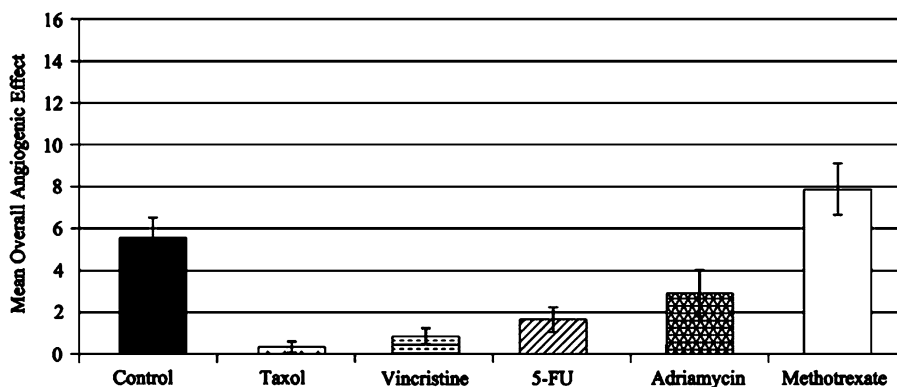


FIG. 4. The effect of antineoplastic drugs on overall angiogenic effect in human breast cancer. Results for each treatment group were determined by calculating the mean angiogenic index (AI) of all wells including those that did not sprout neovessels (i.e., those with an AI \geq 0). Y-error bars indicate ± 2 standard error on the mean (SEM).

reduction in sample sizes resulting from the exclusion of wells that did not exhibit an angiogenic response, the nonparametric Kruskal–Wallis test was used to check for differences among group means for the angiogenic growth data. If the overall test was significant, pairwise comparisons of the treatment groups to the control group were performed using the Wilcoxon rank-sum test with Bonferroni correction factors applied to the nominal level to maintain an overall type I error rate of 0.05.

An additional analysis involving only the control (untreated) wells was performed. In this analysis, overall angiogenic effect means for the four specimens were regressed on time (in days). Weighted linear regression was used since the dependent variable consisted of mean values and not individual measures. Weights were equal to the inverse variance of the means. All statistical analyses were performed using SAS[®] software, version 9.1 (SAS Institute Inc., Cary, NC) and GraphPad Prism, version 4.00

(GraphPad Software, San Diego, CA). Results were considered significant at the 0.05 nominal level.

RESULTS

Four breast cancer specimens were tested against a single concentration of five different antineoplastic drugs. There were an average of 28.6 wells (range 17–60) per treatment group. The mean overall angiogenic effect for the four specimens increased from 0.0 on day 0, to 2.2 (range 0.5–4.0) on day 7, to 5.7 (range 3.3–7.5) on day 14. A positive correlation was noted between mean overall angiogenic effect and days in the assay ($r = 0.84$, $P = 0.0007$) (Fig. 1).

Mean angiogenic initiation (%I) for all four specimens was inhibited most by taxol, 5-FU, and vincristine. Initiation of angiogenesis in these treatment groups was 69%, 66%, and 63% less than control, respectively. Mean %I in the adriamycin group was 39% less than control, while mean initiation in the methotrexate group was 8% greater than control. For three of four (75%) specimens, 5-FU treatment demonstrated statistically less %I than control. Taxol and vincristine treatments yielded less %I in two of four (50%) specimens, while adriamycin treatment yielded less in one of four (25%) specimens. Methotrexate yielded less %I in none (0/4, 0%) of the specimens tested.

Mean angiogenic growth (AI > 0) for all four specimens was inhibited most by taxol, vincristine, and 5-FU. Mean growth in these treatment groups were 81%, 69%, and 46% less than control growth, respectively. Mean growth in the adriamycin group was 17% less than control, while mean growth in the methotrexate group was 5% less than control. Vincristine treatment yielded statistically less growth than control in three of four (75%) specimens. Taxol and 5-FU treatments yielded statistically less growth than control in two of four (50%) specimens. Both adriamycin and methotrexate yielded less growth in none (0/4, 0%) of the specimens tested.

Mean overall angiogenic effect (AI ≥ 0) for all four specimens was inhibited most by taxol, vincristine, and 5-FU. Mean overall effect in these treatment groups was 94%, 89%, and 82% less than control, respectively. Mean overall angiogenic effect in the adriamycin group was 49% less than control, while in the methotrexate group it was 3% greater than control. Taxol, vincristine, and 5-FU treatments yielded statistically less overall effect than control in all four (100%) of the specimens. Adriamycin treatment demonstrated statistically less overall effect than

control in three of four (75%) specimens. Methotrexate yielded statistically less overall effect than control in none (0/4, 0%) of the specimens. On the contrary, statistically more overall angiogenic effect was demonstrated in one of four (25%) specimens treated with methotrexate compared with control.

DISCUSSION

Over the past 20 years, randomized controlled trials have been used to select optimal drug regimens for the treatment of breast cancer. This approach has led to relatively limited increases in breast cancer survival.⁹ Many investigators believe that more rapid progress might come from treating patients individually, i.e., according to their own unique tumor biology rather than “herd” tumor characteristics. The first example of individualizing therapy in breast cancer was made possible through the identification of estrogen receptors (ER) and progesterone receptors (PR). Although hormonal manipulation had been used in management of breast cancer for a century,¹⁰ the identification of these receptors allowed practitioners to select a group of patients most likely to benefit from hormonal therapy. This saved potential nonresponders from the morbidity of hormone ablative procedures and medications.¹¹

More recent advancements in the individualization of breast cancer therapy were developed through the understanding of the HER-2/*neu* oncogene. Transfection studies with DNA from rat neuroglioblastomas initially led to the identification of this gene,¹² and subsequent work done by Slamon et al.¹³ furthered our understanding of it in humans. It is now understood that this gene encodes a transmembrane tyrosine kinase receptor protein that is a member of the human epidermal growth factor receptor family.¹⁴ Overexpression of this receptor is observed in 10–40% of human breast cancers.¹⁵ Tumors that overexpress the HER-2 receptor behave more aggressively and have a worse prognosis.¹³ Understanding of this receptor pathway led to the development of trastuzumab, a humanized monoclonal antibody that specifically binds the extracellular portion of the HER-2 protein receptor. Patients whose tumors overexpress HER-2/*neu* experienced a 25–48% response rate to trastuzumab.¹⁶ This is significantly better than the 0–7% response to trastuzumab seen in patients with less than 3+ expression. By testing for HER-2/*neu* amplification, practitioners can identify a subset of patients that will more likely benefit from treatment with trastuzumab.

While cytotoxic drugs remain the cornerstone of adjuvant treatment in breast cancer, assays that can accurately individualize the selection of chemotherapy regimens are limited. Salmon and Hamburger were the first to develop an assay designed to screen potential chemotherapeutics.¹⁷ They devised an in vitro soft agar culture system capable of supporting clonal growth of a variety of human tumors while suppressing normal cell proliferation. A prospective trial clinically evaluating this assay was done in 1983 by Von Hoff et al.¹⁸ In this trial they compared the clinical responses of patients who received a single reagent either picked by a clinician or picked by the assay. These authors studied patients with many different types of tumors and observed a 25% response rate in those who had received an assay-guided drug versus a 15% rate in patients who were given a clinician-selected reagent.¹⁸ Historically, the chemosensitivity assay has allowed investigators to predict clinical response to single agents with 60–70% accuracy.²

Researchers have developed other human-based chemosensitivity assays as well. Xu et al.¹⁹ used a methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) assay to determine in vitro chemosensitivity. They used the assay results to determine subsequent therapy in patients with breast cancer. Seventy-six percent of patients responded to chemotherapy when it was selected by the MTT assay compared with only 43% when the regimen was determined empirically. There were no statistical differences in survival, however. Other in vitro techniques that have been used to predict chemosensitivity include the subrenal capsule assay,²⁰ the adenosine triphosphate (ATP) bioluminescence assay,²¹ and the capillary cloning system assay.²² In contrast to chemosensitivity assays, Kern and Weisenthal employed a Bayesian statistical model to determine the predictive accuracy of a chemoresistance assay.²³ Their chemoresistance assay exposes specimens to suprapharmacologic concentrations of reagents, and they have shown that drugs failing to suppress tumor growth at these extreme concentrations are likely to fail clinically 90% of the time. Mehta et al. performed a double-blinded retrospective analysis of 96 patients comparing in vitro drug resistance based on this assay to overall survival in patients with breast cancer.²⁴ Survival in patients who received a cyclophosphamide, methotrexate, 5-FU regimen was compared with their in vitro responses to 4-hydroxycyclophosphamide (4HC) and 5-FU. Survival in patients who received doxorubicin plus cyclophosphamide (AC) was compared with their in vitro responses to 4HC and

TABLE 1. Number of specimens with inhibited angiogenesis

	Angiogenic initiation	Angiogenic growth	Overall angiogenic effect
Taxol	3	2	4
Vincristine	3	2	4
5-FU	2	2	3
Adriamycin	1	0	3
Methotrexate	0	0	0

Number of specimens with inhibited angiogenesis. The number of specimens with statistically less angiogenesis compared with control following antineoplastic treatment.

doxorubicin. The authors noted a 45% 5-year survival rate in patients who had high in vitro resistance to both drugs with which they were treated versus an 81% 5-year survival in women who had low in vitro resistance to both drugs with which they were treated.

Although these different assays vary in their methodology, they all share the following characteristic: cytotoxicity of the drug is assessed on the tumor cell itself. To date there are no assays designed to screen chemotherapeutics which consider the antiangiogenic potential of these drugs. This is important because nearly every cytotoxic chemotherapeutic has been shown to demonstrate antiangiogenic effects.^{5,25–28} (Tables 1 and 2). We have previously studied human angiogenesis by plating human tissues in a fibrin–thrombin clot angiogenesis assay.⁶ This assay has enabled the investigator to directly visually assess neovessels sprouting from the cut edge of cancer specimens (Fig. 5). Based on our previous observations we hypothesized that breast cancer specimens would sprout angiogenic blood vessels in a fibrin–thrombin clot angiogenesis assay. We further speculated that we would be able to test the antiangiogenic potential of commonly used cytotoxic drugs against human breast cancer specimens. The specimens tested were fresh 1-mm fragments of tumor that had been recently harvested from patients undergoing surgery for breast cancer. We tested a single dose of five different reagents against malignant breast tumors from four separate patients. Assay results were visually assessed and available within 14 days. Control specimens from all four patients developed neovessels in a time-dependent manner. The angiogenic parameters (initiation of angiogenesis, angiogenic growth, and overall angiogenic effect) of treated specimens were compared with those of control specimens for all four patients. Taxol, vincristine, and 5-FU had the greatest antiangiogenic effects against human neovessel growth in this study while adriamycin and methotrexate had the least.

TABLE 2. *Antiangiogenesis of breast cancer drugs*

Reagent	Our dose	Others' dose	Others' measure of angiogenesis
Taxol	8.6 ng/ml	51–96 pM ²⁵	EC proliferation
Vincristine	1 µg/ml	1.3 ng/ml ²⁶	EC proliferation
5-FU	22.5 µg/ml	5–10 µg/ml ²⁷	EC proliferation
Adriamycin	4 µg/ml	8 µg/ml ²⁸	Chick chorioallantoic membrane
Methotrexate	2.8 µg/ml	20–40 ng/ml ²⁷	EC proliferation

Antiangiogenesis of breast cancer drugs. We used clinically achievable doses of cytotoxic agents in this study. Others' doses refer to preclinical antiangiogenic doses that have been published by other investigators. Also listed is the manner in which they assessed angiogenesis. EC, endothelial cell.

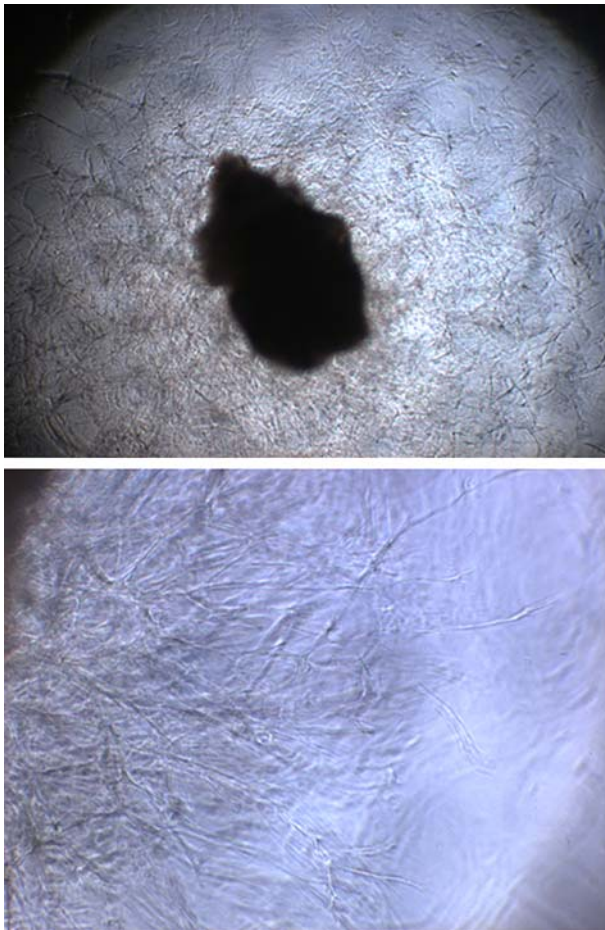


FIG. 5. Photograph of angiogenic blood vessels sprouting from the cut edge of a human malignant tumor in a fibrin–thrombin clot. Images were taken with a Nikon TS-100 inverted microscope (Melville, NY) fitted with a Photometrics Cool Snap cf camera (Roper Scientific Inc, Tucson AZ). Image A, low power (1.7 \times); image B, higher power (4.2 \times).

These results demonstrate that human breast cancer specimens can successfully develop an angiogenic response in a fibrin–thrombin clot angiogenesis assay. In addition, standard cytotoxic drugs will affect angiogenesis in human breast cancer. The interaction

between cytotoxic drugs and angiogenic blood vessels is an element not considered by other human-based chemotherapy screening assays. We believe that these data offer novel and unique information regarding the potential response of cytotoxic drugs in breast cancer. Such information could serve as an additional modality to screen drugs and to identify the most appropriate chemotherapeutic for patients with breast cancer.

Endorsement Disclaimer

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable.

REFERENCES

1. Jørgensen JT, Nielsen KV, Ejlersen B. Pharmacodiagnosics and targeted therapies—a rational approach for individualizing medical anticancer therapy in breast cancer. *Oncologist* 2007; 12:397–405.
2. Salmon SE. This Week's Citation Classic. *Current Contents by ISI* 1984; 46:16.
3. Schrag D, Garewal HS, Burstein HJ, et al. ASCO Working Group on Chemotherapy Sensitivity and Resistance Assays. American society of clinical oncology technology assessment: chemotherapy sensitivity and resistance assays. *J Clin Oncol* 2004;22:3631–8. Epub 2004 Aug 2.
4. Samson DJ, Seidenfeld J, Ziegler K, et al. Chemotherapy sensitivity and resistance assays: a systematic review. *J Clin Oncol* 2004; 22:3618–30.
5. Miller KD, Sweeney CJ, Sledge GW Jr. Redefining the target: chemotherapeutics as antiangiogenics. *J Clin Oncol* 2001;19:1195–206.
6. Woltering EA, Lewis JM, Maxwell PJ IV, et al. Development of a novel in vitro human tissue-based angiogenesis assay to evaluate the effect of antiangiogenic drugs. *Ann Surg* 2003; 237:790–8.
7. Gulec SA, Woltering EA. A new in vitro assay for human tumor angiogenesis: three-dimensional human tumor angiogenesis assay. *Ann Surg Oncol* 2004; 11:99–104.

8. Watson JC, Redmann JG, Meyers MO, et al. Breast cancer increases initiation of angiogenesis without accelerating neovessel growth rate. *Surgery* 1997; 122:509–14.
9. Early Breast Cancer Trialists' Collaborative Group (EBCTCG) Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. *Lancet* 2005; 365:1687–717.
10. Boyd S. On oophorectomy in cancer of the breast. *Br Med J* 1900; 2:1161–7.
11. Jensen EV, Jordan VC. The estrogen receptor: a model for molecular medicine. *Clin Cancer Res* 2003; 9:1980–9.
12. Shih C, Padhy LC, Murray M, et al. Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* 1981; 290:261–4.
13. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235:177–82.
14. Akiyama T, Sudo C, Ogawara H, et al. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986; 232:1644–6.
15. Seshadri R, Figgairi FA, Horsfall DJ, et al. Clinical significance of HER-2/neu oncogene amplification in primary breast cancer. The South Australian Breast Cancer Study Group. *J Clin Oncol* 1993; 11:1936–42.
16. Vogel CL, Cobleigh MA, Tripathy D, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J Clin Oncol* 2002; 20:719–26.
17. Hamburger AW, Salmon SE. Primary bioassay of human tumor stem cells. *Science* 1977; 197:461–3.
18. Von Hoff DD, Clark GM, Stogdill BJ, et al. Prospective clinical trial of a human tumor cloning system. *Cancer Res* 1983; 43:1926–31.
19. Xu JM, Song ST, Tang ZM, et al. Predictive chemotherapy of advanced breast cancer directed by MTT assay in vitro. *Breast Cancer Res Treat* 1999; 53:77–85.
20. Maenpaa JU, Heinonen E, Hinkka SM, et al. The subrenal capsule assay in selecting chemotherapy for ovarian cancer: a prospective randomized trial. *Gynecol Oncol* 1995; 57:294–8.
21. Kurbacher CM, Cree IA, Bruckner HW, et al. Use of an ex vivo ATP luminescence assay to direct chemotherapy for recurrent ovarian cancer. *Anti-cancer Drugs* 1998; 9:51–7.
22. Von Hoff DD, Sandbach JF, Clark GM, et al. Selection of cancer chemotherapy for a patient by an in vitro assay versus a clinician. *J Natl Cancer Inst* 1990; 82:110–6.
23. Kern DH, Weisenthal LM. Highly specific prediction of anti-neoplastic drug resistance with an in vitro assay using supra-pharmacologic drug exposures. *J Natl Cancer Inst* 1990; 82:582–8.
24. Mehta RS, Bornstein R, Yu IR, et al. Breast cancer survival and in vitro tumor response in the extreme drug resistance assay. *Breast Cancer Res Treat* 2001; 66:225–37.
25. Bocci G, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. *Cancer Res* 2002; 62:6938–43.
26. Iwahana M, Utoguchi N, Mayumi T, et al. Drug resistance and P-glycoprotein expression in endothelial cells of newly formed capillaries induced by tumors. *Anticancer Res* 1998; 18:2977–80.
27. Cwikiel M, Eskilsson J, Albertsson M, et al. The influence of 5-fluorouracil and methotrexate on vascular endothelium. An experimental study using endothelial cells in the culture. *Ann Oncol* 1996; 7:731–7.
28. Maragoudakis ME, Peristeris P, Missirlis E, et al. Inhibition of angiogenesis by anthracyclines and titanocene dichloride. *Ann N Y Acad Sci* 1994; 732:280–93.